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## Red Wine and Pomegranate Extracts Suppress Cured Meat Promotion of Colonic Mucin-Depleted Foci in Carcinogen-Induced Rats

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### ABSTRACT

Processed meat intake is carcinogenic to humans. We have shown that intake of a workshop-made cured meat with erythorbate promotes colon carcinogenesis in rats. We speculated that polyphenols could inhibit this effect by limitation of endogenous lipid peroxidation and nitrosation. Polyphenol-rich plant extracts were added to the workshop-made cured meat and given for 14 days to rats and 100 days to azoxymethane-induced rats to evaluate the inhibition of preneoplastic lesions. Colons of 100-d study were scored for precancerous lesions (mucin-depleted foci, MDF), and biochemical end points of peroxidation and nitrosation were measured in urinary and fecal samples. In comparison with cured meat-fed rats, dried red wine, pomegranate extract,  $\alpha$ -tocopherol added at one dose to cured meat and withdrawal of erythorbate significantly decreased the number of MDF per colon (but white grape and rosemary extracts did not). This protection was associated with the full suppression of fecal excretion of nitrosyl iron, suggesting that this nitroso compound might be a promoter of carcinogenesis. At optimized concentrations, the incorporation of these plant extracts in cured meat might reduce the risk of colorectal cancer associated with processed meat consumption.

### Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide and the second cause of cancer death in affluent countries (1). Epidemiological studies show that processed meat intake is linked to the risk of CRC (2). The World Cancer Research Fund panel considers this risk as convincing and recommends avoiding processed meat consumption (3,4). The World Health Organization classified consumption of processed meat as “carcinogenic to humans” (IARC Group 1) based on sufficient evidence for colorectal cancer (5). Making safer meat products could be an alternative to banning cured meat (6,7). In carcinogen-initiated rats, given a low-calcium diet freeze-driedcooked ham and moist hot-dog increase significantly the number of mucin-depleted foci (MDF) (8,9). The intake of an experimental cured pork meat, similar to an air-exposed cooked shoulder-ham (DCNO for dark cooked meat with nitrite, oxidized, described below), also promotes carcinogenesis in rats (10). In human volunteers, cured meat intake increases endogenous nitrosation and fat peroxidation and fecal water-induced oxidative DNA damage (7,11).

We have speculated that heme-iron could explain in part the promoting effect of processed meat (12,13), and added experimental support to this hypothesis: Dietary heme (free heme stabilized by a chloride ion) promotes azoxymethane-induced aberrant crypt foci (ACF) in the colon of rats (14). Heme, but not hemoglobin, mimics

the effect of ham on biomarkers associated with carcinogenesis (8).

Heme-iron catalyzes the formation of apparent total nitroso compounds (ATNC) (15) and of lipid peroxidation end products, e.g., 4-hydroxynonenal and other alkenals (16). 4-hydroxynonenal is cytotoxic and genotoxic to the intestinal epithelial cells (17,18). Potentially carcinogenic ATNC are formed in the gastrointestinal tract by N-nitrosation of peptide-derived amines or amides. Nitrosylated heme-iron present in processed red meat also represents a significant part of measured ATNC (13,19,20). ATNC and alkenals could explain tumor promotion by dietary heme and by cured meat (6,21,22).

Our starting hypothesis here was that lipid peroxidation end products would promote carcinogenesis (23), and that polyphenols would decrease heme-induced luminal peroxidation (24) and hence carcinogenesis. There is ample evidence that polyphenols and plant extract can block heme-induced fat peroxidation: For instance, quercetin, red wine, and  $\alpha$ -tocopherol suppress myoglobin-induced peroxidation in a fat/water emulsion that mimics the gastric environment and blocks the accumulation of conjugated dienes (25,26). In human volunteers, red wine polyphenols strongly decrease postprandial plasma malondialdehyde after a red meat meal, probably by suppressing heme-induced peroxidation in the stomach (27–29). In rats, a mix of rutin and butylated hydroxyanisole inhibits heme-induced lipid peroxidation in the gut and suppresses carcinogenesis promotion (14). In addition, polyphenols

such as punicalagin and ellagic acid from pomegranate can chelate iron through catechol groups (30,31), while propyl gallate, tannic acid, thymol, vanillin, and ascorbate and  $\alpha$ -tocopherol can inhibit nitrosation and ATNC formation (32,33). The present study was designed to test the hypothesis that polyphenols can prevent the promotion of colon tumorigenesis by processed meat, by suppressing lipid peroxidation in the gut. In a short-term screening study, several agents were added to DCNO cured meat during the manufacturing process. Such diets were given to rats for 14 days. Early lipid peroxidation end points were measured in feces and urine. Most promising agents selected during these screening studies were added to DCNO and tested for chemoprevention in a 100-day carcinogenesis study in rats. Tumorigenesis end points were azoxymethane-induced preneoplastic lesions (ACF and MDF) in rats. The results showed that dried red wine, pomegranate extract, and  $\alpha$ -tocopherol prevented meat-associated formation of fecal nitrosyl iron and promotion of preneoplastic lesions.

## Materials and Methods

### Animal Study Design

Two sequential studies were performed on male Fischer 344 rats purchased at 4–5 weeks of age from Charles River (St Germain l'Arbresle, France): A 14-day study investigated the effect of plant extracts added to an experimental cured meat on early fecal and urinary biomarkers in rats. A 100-day study measured the antipromoting effect of four plant extracts added to the same cured meat, on preneoplastic lesions in carcinogen initiated rats. Animal care was in accordance with the guidelines of the European Council on animals used in experimental studies. Study was done in an accredited animal colony (French A 31504) by approved staff (e.g., P.I. Corpet: Certificat d'autorisation d'expérimenter sur animaux vertébrés vivants #31-121).

### Short-term Study Design (14 Days-long)

Forty-three rats were housed individually in metabolic cages. They were kept at 22°C and 12 h–12 h light/dark cycle. After 3 days of acclimatization to the animal colony and to a standard AIN76 diet, rats were randomly allocated to eight groups. There were five rats in each experimental group given DCNO cured meat with plant extracts (described below), and eight rats in the control group fed DCNO. Rats were fed the experimental diets described below during 14 days and allowed free access to tap water. Body weight was monitored every week. Food and water intakes were measured at day 13. Feces and urine were collected at days 11 and 12 and frozen at  $-20^{\circ}\text{C}$ . Animals were terminated by  $\text{CO}_2$  asphyxiation on day 14. Fecal water samples (preparation described below) were analyzed for heme, cytotoxicity, and thiobarbituric acid reactive substances (TBARS). Urine samples were analyzed for 1,4-dihydroxynonane mercapturic acid (DHN-MA).

### Carcinogenesis Study (100 Day-long): Animals and Design

Eighty-six rats were housed individually in stainless steel, wire-bottomed cage (same animal colony as above). After 7 days of acclimatization, each rat received a single i.p. injection of azoxymethane (20 mg/kg i.p.; Sigma Chemical) in NaCl (9 g/L). Seven days later, they were randomly allocated to seven groups (N D 10 rats per group, except control group, N D 26) and fed the experimental DCNO-based diets described below. Body weights were monitored every week for four weeks, then every two weeks. Food and water intakes were measured at days 20 and 80. Feces were collected daily between days 18 and 21, and 80 and 91 and frozen at  $-20^{\circ}\text{C}$ . Between days 74 and 76, each rat was put in a metabolic cage, and urine was collected and frozen at  $-20^{\circ}\text{C}$ . Rats were killed by  $\text{CO}_2$  asphyxiation in a random order at day 96–98. Colons were removed and fixed in 10% buffered formalin (Sigma Chemical) between two sheets of filter paper with a blinding code. ACF and MDF were scored. Fecal water samples were analyzed for heme, TBARS, cytotoxicity, and ATNC. Urine samples were analyzed for DHN-MA.

### Animal Diets

The type of meat and the additives that were given to groups of rats during the short-term and carcinogenesis studies are listed in the first column of Tables 1 and 2, respectively.

Pork meat was cured in a specialized workshop by IFIP-Institut du Porc (14-day study) and in a ham factory by Fleury Michon (Pouzauges-France) (100-day study). Meat was given as such (moist piece) to the rats because freeze-drying boosts peroxidation of fat in meat (34). The experimental cured meat, which was similar to air-exposed picnic ham and called DCNO, was chosen because it promotes carcinogenesis in rats (10). DCNO was made from *Musculus vastus intermedius*, cured with 2.19 g salt with 0.6% sodium nitrite (131 ppm  $\text{NaNO}_2$ ), and 1.4 g sodium erythorbate (an ascorbate isomer) per 100 g meat. DCNO was then heated at  $70^{\circ}\text{C}$  for 3 h in vacuum-sealed plastic bags in a water bath. The final product contained 12 mg heme-iron/kg, 71 mg sodium nitrite/kg, and 500 mg ascorbate/kg. One group of rats was given an erythorbate-free DCNO. The processed meat was divided into 1.3-cm thick slices of 300 g, that were stored separately at  $-20^{\circ}\text{C}$  in air-tight plastic bags with low-oxygen permeability (14 day study) or under  $\text{CO}_2/\text{N}_2$  50/50 atmosphere to avoid further fat oxidation (100 day study). Before being given to rats, each slice was exposed to air for five days in a dark refrigerator ( $4^{\circ}\text{C}$ ), then cut into ten 30-g portions that were given to rats at 5:00 p.m. for 14 or 100 days. A low-calcium powdered diet (35) was given in a separated feeder, 7.6 g/d/rat, so that each rat would eat roughly half meat/half powder (dry matter). This modified AIN76 diet was prepared by UPAE (INRA, Jouy, France) as follows (g/100 g):

sucrose, 59.5; corn starch, 15.0; cellulose, 12.5; AIN76 mineral mix without calcium, 8.7; AIN76 vitamin mix, 2.5; methionine, 0.75; calcium phosphate, 0.52; choline bitartrate, 0.5. Safflower oil (5 g) was mixed with 100-g powder to provide polyunsaturated fatty acids (MP Biomedicals, Illkirch, France).

Six polyphenols-rich plant extracts were added to DCNO during the curing process, at a concentration recommended by the supplier: white grape extract (NutriPhy\_ white grape 100, 72% of total polyphenols, CHR Hansen, Horsholm, Denmark; 0.055% w/w in DCNO), carnosic acid (StabilEnhance\_ OSR5 extracted from rosemary leaves, 10% carnosic acid, Naturex, Avignon, France; 1% w/w in DCNO), and a water soluble rosemary extract, containing 7% of rosmarinic acid (Stabilenhance\_ WSR6, Naturex; 0.66% w/w in DCNO), red wine concentrate, 10% of total polyphenols (Avvin9005\_, Diana Naturals, Antrain, France; 2% w/w in DCNO), pomegranate extract, 12% ellagic acid (Naturex, Ultimate Botanical Benefits; 0.6% w/w in DCNO), green tea extract, 98% of total polyphenols (Naturex; 0.08% w/w in DCNO). Polyphenol data were given by the suppliers, and the composition of extracts was not determined more precisely in this pilot study. Another group was given DCNO supplemented with  $\alpha$ -tocopherol (Covitol\_, Nutrition & Health, Cognis, BASF; 0.045%): this fat-soluble antioxidant agent suppresses MDF in carcinogen-induced rats and was used as a positive control for protection (7). A last group of rat given DCNO without sodium erythorbate was added to the carcinogenesis study.

### Meat Composition

Processed meat was analyzed by Lareal (Vannes, France, laboratory specialized in physicochemical and microbiological analyzes) for total iron, total pigments, nitrosylated pigments (36). Hexanal, a marker of secondary products of lipid peroxidation, was analyzed by Lareal by gas chromatography of the headspace of the sample dispersed in phosphate buffer at 37°C, with solid-phase micro-extraction fiber. Trolox equivalent antioxidative capacity (TEAC-1), malondialdehyde (MDA by HPLC), and TBARS (after acidic extraction) were measured by ADIV (Clermont-Ferrand, France). The oxygen radical absorbance capacity (ORAC) was measured by Naturex (Avignon, France). Two measures per processed meat batch were done.

### Fecal and Urinary Measures

#### Analysis of Heme, Thiobarbituric Acid Reactive Substances in, and Cytotoxicity of Fecal Water, and 1,4-Dihydroxynonane Mercapturic Acid in Urine

Fecal pellets were collected under each cage for 24 h, at day 11 of the short-term study and days 88–91 of the carcinogenesis study. TBARS value was used as a global measure of lipid peroxidation end products. Fecal water was prepared, and heme and TBARS were measured in fecal water exactly as previously described (7) except that

1 mL of distilled water was added to 0.42 g of crushed fresh feces, but not to 0.3 g of dried feces. 1,4-Dihydroxynonane mercapturic acid (DHN-MA) is the main urinary metabolite of 4-hydroxynonenal, which is a major toxic end product of endogenous fat peroxidation (16). The 24-h urine was collected under each metabolic cage, at day 11 of the short-term study and days 74–76 of the carcinogenesis study. DHN-MA assay was done (n D 5–8 for the 14-day study and n D 10–26 for the 100-day study) as previously described (7). To determine cytotoxicity of fecal water (n D 6), the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used on a cancerous mouse colonic epithelial cell line, CMT93 (European Collection of Animal Cultures), as previously described (7).

### ATNC Analysis

ATNC were analyzed using a modification of the method previously used (37), using a CLD88 Exhalyzer (Ecomedics, Duernten, Switzerland). Sulfamic acid solution (500 mL, 5%) was added to 100 mL of fecal water to remove nitrite, and samples were injected into a purged vessel kept at 60°C and filled with a standard tri-iodide reagent (38 mg I<sub>2</sub> was added to a solution of 108 mg KI in 1 mL water; to this mixture, 13.5 mL of glacial acetic acid was added) to determine total ATNC. To determine mercury(II) stable compounds, 100 mL of 10-mM aqueous HgCl<sub>2</sub> was added prior to analysis; to determine mercury(II) and ferricyanide stable compounds, 100 mL each of 10 mM aqueous HgCl<sub>2</sub> and 10-mM aqueous K<sub>3</sub>Fe(CN)<sub>6</sub> solution were added prior to analysis. Nitrosothiols were determined as the difference between total ATNC and mercury(II) stable ATNC; nitrosyl iron was determined as a difference between mercury(II) stable ATNC and mercury(II) and K<sub>3</sub>Fe(CN)<sub>6</sub> stable compounds. Data are concentrations (in mM), measured in triplicate in 100 mL of each sample.

### ACF and MDF Assays

ACF and MDF were scored by a single observer blinded for the origin of the colon, exactly as described previously (7). Number of lesions and number of crypts per lesions (i.e., size of ACF and MDF) were numbered.

### Statistical Methods

Results were analyzed using Systat 10 software for Windows, and all data were reported as mean  $\pm$  SD (except Fig. 1B). Values were considered firstly using one-way analysis of variance. If a significant difference was found between all groups ( $P < 0.05$ ), comparison of each experimental group with the control group was made using Dunnett's test. For ORAC analysis, data show results of two measures per processed meat batch, but Student t-test statistics could be done because the within-pair correlation was high; however,  $P$  values should be taken cautiously (38).



## Results

### Fourteen-Day Study: Effect of Plant Extracts on Peroxidation Biomarkers

#### Fecal and Urinary Fat Peroxidation Biomarkers

Dietary DCNO cured meat increases the number of carcinogen-induced precancerous lesions, and urinary and fecal water peroxidation biomarkers in rats (10,14). These early peroxidation biomarkers were thus measured here because they correlate with heme-induced promotion of colon carcinogenesis (8,39). Extracts of pomegranate, red wine, white grape, green tea, rosemary, and carnosic acid and  $\alpha$ -tocopherol were added to DCNO before being fed to rats for 14 days. As shown in Table 1, fecal water from rats given DCNO added with pomegranate, red wine, or white grape extracts contained half TBARS than control rats given DCNO. In contrast, fecal water from rats given DCNO plus carnosic acid contained surprisingly twice more TBARS than controls. All tested extracts led to some reduction in urinary DHN-MA, but only rosemary extract significantly decreased the excretion of this 4-hydroxynonenal metabolite. All the tested plant extracts reduced fecal heme excretion in DCNO-fed rats, except for  $\alpha$ -tocopherol and rosemary extract. Finally, addition of plant extracts in DCNO did not affect the cytotoxicity associated with DCNO consumption.

#### Choice of Polyphenol Additives for the Carcinogenesis Study

Pomegranate, red wine, and white grape extracts that decreased TBARS in fecal water of DCNO-fed rats (Table 1) were chosen to be tested in the carcinogenesis study because our starting hypothesis was that polyphenols would exert their protective action by inhibiting lipid peroxidation (25,40). We also chose to test carnosic acid, a common additive to brine in Europe, because it surprisingly increased TBARS in fecal water. In addition, we tested  $\alpha$ -tocopherol as a protection control because it suppresses cured meat promotion in rats (7). Finally, a special DCNO meat, cured without erythorbate, was given to a group of rat to test the effect of this common additive.

### Carcinogenesis Study: Effect of Plant Extracts

#### General Observation

All rats survived and were healthy, except rats given carnosic acid that had diarrhea. Moist meat and powdered diet were given to the rats in separated feeders: the relative intake of meat and of powder that was 48:52 (dry weight) on day 18 of the study slowly changed to 39:61 on day 82. The final body weight of rats was  $343 \pm 19$  g without significant difference between groups except rats fed cured meat plus carnosic acid ( $321 \pm 16$  g,  $P < 0.05$ ). Rats in this group ate and drank less than the rats in others groups: their average food intake per day was  $12 \pm 1$  g compared with  $13 \pm 1$  g in other groups ( $P < 0.05$ ). Water intake was reduced in rat fed carnosic acid and increased

in rat fed  $\alpha$ -tocopherol or white grape extract, compared with the other groups (full data not shown,  $P < 0.0001$ ).

#### Quantification of ACF and MDF

A DCNO-based diet increases the number of MDF and ACF in the colon of carcinogen-injected rats, in comparison with a no-meat control diet (7,10). The DCNO diet was thus chosen as a promoting control to test potentially protective plant extracts. At the doses tested, all plant extracts decreased the number of MDF per colon in comparison with DCNO diet, but only  $\alpha$ -tocopherol, pomegranate, and red wine extracts led to a significant protection (Fig. 1B). Neither the number of ACF nor the MDF and ACF multiplicity was different between groups (Table 3). Surprisingly, the removal of erythorbate from DCNO curing brine led to a significant reduction in the number of colonic MDF. Mean number of large ACF or of large MDF, with 4 or more crypts per foci, was similar in all dietary groups. In an attempt to explain the observed protection, diets, fecal water, and urine were analyzed for lipoperoxides and nitroso compounds.

#### Meat Analyses

Trolox, MDA, TBARS, ORAC, hexanal, and nitrosylated heme were analyzed, but only ORAC, hexanal, and nitrosylated heme values were reported here, because they are not significantly affected by the modification of process of meats. ORAC is a measure of antioxidant power. As expected, all tested plant extracts increased cured meat ORAC value 2–3 times and suppressed hexanal production, a measure of meat peroxidation (Table 4). Heme and NO from nitrite can form nitrosyl heme (19) that might be the promoting factor in cured meat (8). The tested plant extracts did not change much nitrosylated heme concentration in meat, except red wine that increased it (Table 4).

#### Fecal and Urinary Fat Peroxidation Biomarkers

Fecal water from rats fed DCNO added with pomegranate, red wine, or white grape extracts, or  $\alpha$ -tocopherol contained 1.5–2 times less TBARS, and about 1.5 times less heme than fecal water from rats fed DCNO alone (Table 2). Pomegranate extract, carnosic acid, and  $\alpha$ -tocopherol also decreased urinary DHN-MA, a metabolite of 4-hydroxy-nonenal. Carnosic acid that significantly increased fecal TBARS in the first study (Table 1) tended to increase it in this second study (not significant).

Addition of plant extracts had not modified the cytotoxicity of fecal water in DCNO group except for carnosic acid that induces a significant increase in fecal water cytotoxicity (Table 2). Surprisingly, the absence of erythorbate in DCNO significantly decreased fecal TBARS and heme compared with erythorbate-supplemented DCNO, without modification of cytotoxic activity of fecal water (Table 2).

#### Fecal Nitroso Compounds

ATNC concentration in fecal samples was reduced by the addition of a plant extract to the curing brine of DCNO

(Fig. 1A). The reduction was more than a three-fold (except white grape). Significance could not be formally established since only one value was obtained per group because feces from all rats in one dietary group had been pooled, so results were interpreted with caution. "ATNC" are a complex mixture of nitrite-derived products, and the ATNC composition was not identical in the feces from different groups. Fecal ATNC from rats fed DCNO cured meat plus carnosic acid or white grape extract were made of 100% nitrosyl iron (Fig. 1A). In contrast, fecal ATNC from rats fed DCNO with pomegranate or red wine extracts were 100% nitrosothiols (data not shown). Tocopherol fully suppressed nitrosation, but the removal of erythorbate from DCNO curing brine led to a fifty percent increase in fecal ATNC, no nitrosyl iron being detected (Fig. 1A).

## Discussion

This study shows that polyphenol-rich plant extracts can inhibit the promotion of colonic mucin-depleted foci by cured meat that had been demonstrated repeatedly in this model (7–10): dried red wine and pomegranate extract suppressed cured meat-induced colon tumorigenesis promotion as well as  $\alpha$ -tocopherol, while white grape extract and carnosic acid extracted from rosemary did not. Promotion was evidenced on a surrogate end point biomarker, mucin-depleted foci. MDF, formed by dysplastic crypts devoid of mucin, have been identified in the colon of humans at high risk for colon cancer (41). Like tumors, MDF harbor mutations in genes affecting colon carcinogenesis (Apc and K-ras) and show Wnt signaling activation (42), a dramatic reduction of MUC2 expression (43), and a strong activation of the inflammatory process (44), all features suggesting that MDF are precancerous. Several rodent studies suggest that MDF are better predictors of colorectal cancer than ACF are (45), and respond more consistently than ACF to promotion by red and processed meat and by dietary heme (9,20,39); this is why we focused on MDF data.

The promotion of colon carcinogenesis by fresh, moist cured meat (DCNO) in rats has been associated with increased fecal nitroso-compound (ATNC) concentrations and increased fecal biomarkers of fat peroxidation (TBARS) (10). Hence, we chose to use DNCO to identify prevention strategies aiming at normalizing fecal biomarkers. However, the prediction of cancer-promoting properties in food by simple chemical analysis would be a great step toward cancer prevention. Unfortunately, no correlation was seen between cured meat composition and the number of MDF: neither hexanal, ORAC, nitrosylated heme, nor any other meat component was associated with MDF promotion. This supports the hypothesis that CRC promotion by processed meat is not directly due to a factor present in food. Meat-induced endogenous factors would thus promote MDF, e.g., aldehydes or N-nitroso compounds (6). Our starting hypothesis was that lipid peroxidation end products would promote carcinogenesis, and that polyphenols would

decrease luminal peroxidation. Polyphenols can scavenge oxygen radicals, preventing the damage toward macromolecules and peroxidation of fatty acids, and they can bind iron, thus reducing catalytic properties of heme (46). The measurement of ORAC is commonly used to study the radical-scavenging ability of polyphenols. Here, the tested plant extracts doubled or tripled the ORAC of meat (Table 4). In addition, fecal excretion of heme-iron was reduced in rats given polyphenol-supplemented meat (Table 2). However, neither the antioxidant effect nor the reduced fecal heme-iron was linked with MDF reduction: for instance, carnosic acid tripled ORAC value in meat but did not reduce MDF number in rats (Fig. 1B).

Our previous studies strongly suggest that fecal aldehydes collectively measured by the TBARS assay would participate to carcinogenesis promotion in meat-fed rats (8,39). Present data support this hypothesis because all plant extracts that decreased MDF number significantly reduced fecal TBARS concentration. In this way, previous in vitro data of our team allowed to propose that a premalignant cell selection by heme-induced aldehydes explains the heme-induced promotion of MDF (20). Thus, limitation of peroxydation and aldehydes formation by antioxidant could explain the protective effect by limitation of the selection of preneoplastic cells. In addition, carnosic acid that increased TBARS did not reduce MDF number (Tables 1 and 4, Fig. 1B). In contrast, white grape extract reduced fecal TBARS but had no effect on MDF number. This discrepant group may suggest that TBARS are not the only parameter involved in CRC promotion.

Although data on ATNC were obtained on fecal pools, our results support the hypothesis of Cross et al on the role of endogenous ATNC in the promotion of CRC by processed meat. The presence of nitrosyl iron in feces, but not the other types of ATNC, was associated with the promotion of CRC by cured meat (Fig. 1). Several human studies strongly suggest that the formation of ATNC can explain the positive links between processed meat intake and CRC (10,11,47). Here,  $\alpha$ -tocopherol fully inhibited the formation of fecal ATNC and suppressed MDF promotion in rats. Similarly, the reduction of MDF by red wine and pomegranate extracts was associated with reduced fecal ATNC and lack of nitrosyl iron in feces (Fig. 1). Nitrosyl iron was indeed the only fecal marker that was consistently associated with MDF promotion. However, no dose-response relationship was seen, since white grape and carnosic acid that boosted nitrosyl iron formation did not increase the MDF number over the control number (Fig. 1). We nevertheless suggest that nitrosyl iron might be used as a short-term biomarker to screen additives added to cured meat to reduce cancer risk.

To test the hypothesis that nitrosation can explain promotion, an artificial model of cured meat was made with no erythorbate. Currently, all commercial processed

meats contain erythorbate. Indeed, this additive is usually added to brine during the curing process to increase nitrosylation and to block nitrosation (19). As expected, fecal ATNC value was higher in rats given erythorbate-free DCNO than in control rats given DCNO with erythorbate (C55%, Fig. 1A). Surprisingly, this ATNC increase was associated with a decrease in the number of MDF per colon, which shows that all ATNC types do not promote tumorigenesis. However, no nitrosyl iron was detected in rats given erythorbate-free DCNO (Fig. 1A), as already observed by Hotter, Zhou and Mirvish (Abstract B-111, *Frontiers in Cancer prevention*, Am. Ass. Cancer Res., Boston, Oct. 2011). We thus suggest a central role of luminal nitrosyl iron in the promotion of colorectal tumorigenesis by cured meat, associated with a minor role of luminal aldehydes. This contradicts Hogg's hypothesis that the sequestration of the "nitrosating potential" of diet as nitrosyl iron is a protective mechanism (48). In contrast, it supports Kuhnle's hypothesis that nitrosyl heme may cause the formation of DNA adduct O6-carboxymethyl guanine in colonic cells (49), which are found in stools of volunteers given red meat (37) with a stimulating effect of heme-iron on adduct production during in vitro fermentation of meat (50). The evidence presented here is weak, however, since no statistics could be done on ATNC data, and because no dose-effect relation was seen between nitrosyl heme excretion and MDF numbers (Fig. 1). Major weaknesses of this study are the pooling of fecal samples before ATNC analysis and the lack of a no-meat arm. Hence, no statistics could be done on ATNC data: significance of the three-fold reduction in total ATNC by plant extracts, and of full suppression of nitrosyl iron by three of the extracts is unknown. In addition, the present study was not designed to confirm MDF promotion by cured meat (DCNO). This promoting effect had repeatedly been shown in the same model (7–10), but could not be tested again in the present study.

The finding that it is possible to counteract the cancer-promoting effect of processed meat by adding selected plant extracts into the meat should have consequences on public health and on dietary recommendations.

The World Cancer Research Fund's advice to avoid processed meat may be updated with the advice that any cured meat meal should also include a polyphenol- or tocopherol-rich plant food. Despite recommendations, individuals, particularly those in low socioeconomic groups, consume large amounts of processed meat. These people are at a higher risk of CRC, early disability, and death. We suggest that the meat industry should use specific protective plant-based additives during the curing process, as this could reduce cancer risk in all consumers. Making safer meat products might be a better approach than banning meat (6,7).

## Conclusions

This study shows that the incorporation of polyphenol rich plant extracts (pomegranate or red wine) or of  $\alpha$ -tocopherol inhibited the promoting effect of cured meat on preneoplastic lesions in carcinogen-induced rats. If these results were confirmed in volunteers' study, these agents might be added to meat during the curing process to make functional processed meat. This study represents an informative starting point; however, future research should address dose dependence and potential efficacy of modified meats that might induce effects ranging from protection, lack of protection to possible cancer-promoting effect at other doses. The use of the protective agents would reduce colorectal cancer risk compared with processed meat. This study also shows that fecal excretion of a specific class of nitroso compounds, nitrosyl iron was associated with tumorigenesis promotion by cured meat.

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## Declaration of Interest

G. Nassy and J. L. Vendeuvre were employed by the Institut Français du Porc (IFIP).

N. Bastide, N. Naud, S. Taché, F. Guéraud, D. Hobbs, G. Kuhnle, D. E. Corpet, F. H. F. Pierre: No conflicts of interest.

## Author Contributions

N. Bastide and N. Naud contributed equally to this work; F. H. F. Pierre, D. E. Corpet, G. Nassy, and J. L. Vendeuvre designed research; Bastide, Naud, S. Taché, F. Guéraud, D. A. Hobbs, and G. Kuhnle conducted research; Bastide, Corpet, and Pierre analyzed data and wrote the paper; Corpet and Pierre had primary responsibility for final content. All authors have read and approved the final manuscript.

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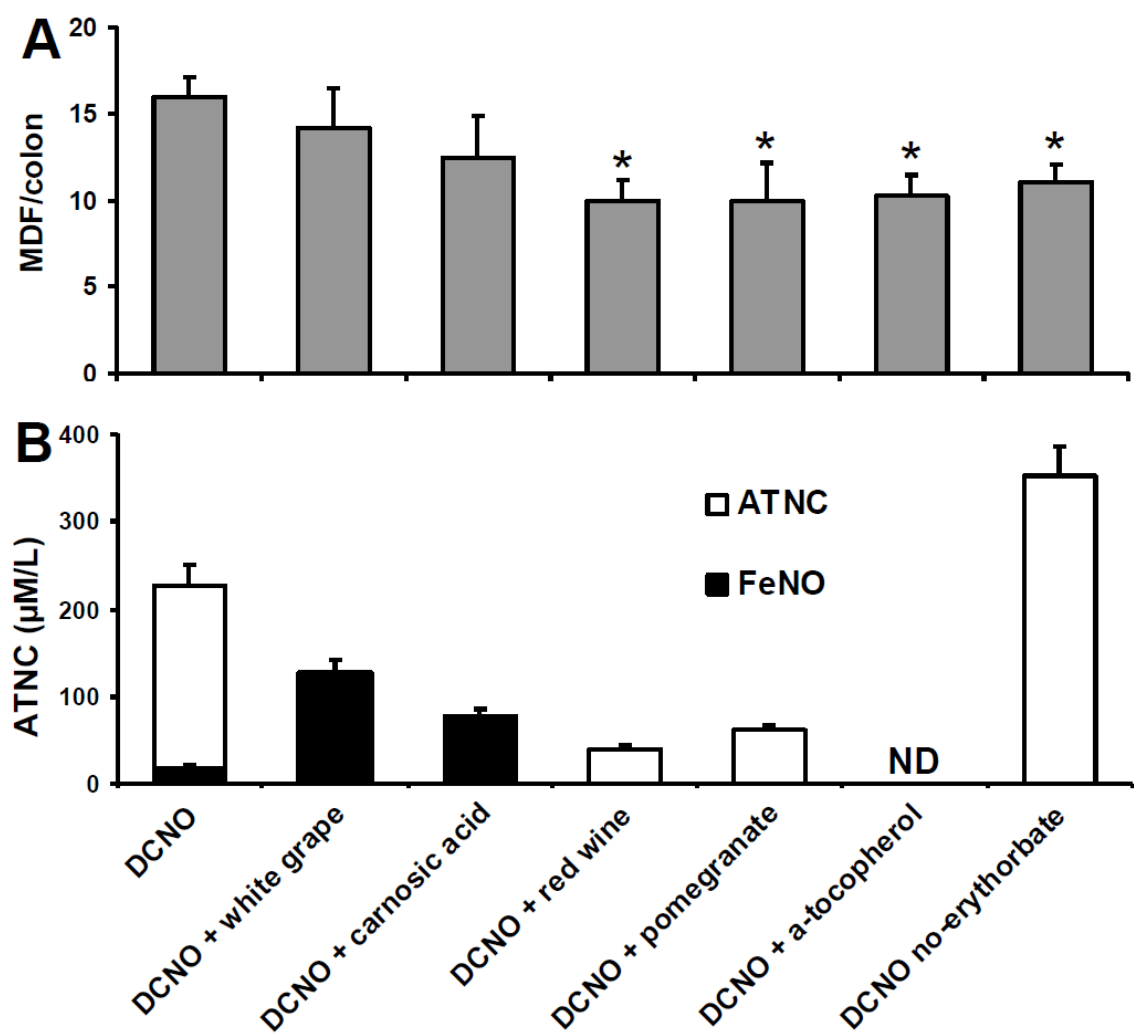


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**Figure 1: Faecal excretion of nitroso compounds and promotion of preneoplastic lesions in the colon of rats.**

A: Mucin Depleted Foci (MDF) in the colon of azoxymethane-initiated rats given cured meat added with plant extracts for 100 days. Values are mean  $\pm$  SEM (same data in Table 3). \* Significantly different from DCNO by Dunnett's t test.

B: Apparent Total N-nitroso Compounds (ATNC) and nitrosyl iron (FeNO) values were obtained on pooled faecal samples from all rats in one group: error bars show analytical SD. ND: not detected.



**Table 1. Faecal and urinary biomarkers in rats given cured meat with plant extracts during the fourteen-day study**

Diet	N. of rats	Haem in FW (nmol/24h)	TBARs in FW (Eq. MDA nmol/24h)	Urinary DHN-MA (ng/24h)	Cytotoxicity of FW (% of dead cells)
DCNO	8	279±90	138±46	410±210	22±8
DCNO + White grape	5	116±54**	77±23*	287±89	23±24
DCNO + Carnosic acid	5	140±72**	273±81***	258±83	29±13
DCNO + Rosemary	5	196±104	144±18	222±30*	20±19
DCNO + Red wine	5	174±70*	68±38**	262±53	35±11
DCNO + Pomegranate	5	97±38***	64±35**	307±225	37±5
DCNO + Green tea	5	150±56*	87±21	288±128	35±13
DCNO + $\alpha$ -tocopherol	5	193±130	98±56	370±194	12±16

FW: faecal water. TBARS: thiobarbituric acid reactive substances; MDA: malondialdehyde; DHN-MA: dihydroxynonene mercapturic acid; DCNO: dark meat, cooked, cured with sodium nitrite, oxidized by air.

Significantly different from DCNO by the Dunnett's t test: \* P < 0.05; \*\* P < 0.01; \*\*\* P < 0.001.

**Table 2. Effect of cured meat diets on fecal and urinary biomarkers in carcinogen-initiated rats 80 days after an azoxymethane injection**

FW : fae cal wat er. TB AR S: thio bar	Diet	N. of rats	Haem in FW (nmol/24h)	TBARs in FW (Eq. MDA nmol/24h)	Urinary DHN-MA (ng/24h)	Cytotoxicity of FW (% of dead cells)
	DCNO	26	238±84	153±16	243±99	52±20
	DCNO + White grape	10	145±40***	99±19***	235±80	57±32
	DCNO + Carnosic acid	10	135±61***	164±39	122±34***	100±1***
	DCNO + Rosemary	10	172±51**	76±11***	191±60	59±16
	DCNO + Red wine	10	162±55**	85±11***	172±64**	66±17
	DCNO + Pomegranate	10	135±41***	120±19**	124±44***	46±20
	DCNO + Green tea	10	162±46**	132±14**	257±90	47±23

bituric acid reactive substances; MDA: malondialdehyde; DHN-MA: dihydroxynonene mercapturic acid; DCNO: dark meat, cooked, cured with sodium nitrite, oxidized by air.

Significantly different from DCNO by the Dunnett's t test: \* P < 0.05; \*\* P < 0.01; \*\*\* P < 0.001.



**Table 3** Preneoplastic lesions (ACF and MDF) in the colon of rats fed cured meat added with plant extracts for 98 d, 105 d after an azoxymethane injection<sup>1</sup>

Diet	No. of rats	MDF/Colon		Crypt/MDF		ACF/colon		Crypt/ACF	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
DCNO	26	16.0	5.8	2.6	0.8	106	22	3.4	0.2
DCNO + White grape	10	14.2	7.3	2.3	0.2	105	14	3.4	0.2
DCNO + Carnosic acid	10	12.5	7.4	2.6	0.7	95	17	3.5	0.2
DCNO + Red wine	10	10.0	3.5**	2.6	0.5	108	19	3.4	0.3
DCNO + Pomegranate	10	10.0	6.8**	2.3	0.4	108	28	3.6	0.2
DCNO + $\alpha$ -tocopherol	10	10.3	3.6**	2.4	0.4	99	21	3.4	0.3
DCNO without erythorbate	10	11.1	3.1*	2.6	0.5	103	24	3.5	0.3

<sup>1</sup> ACF: aberrant crypt foci. MDF: mucin depleted foci. Other notes: see Table 1.

**Table 4** Processed meat analysis: Antioxidant activity, nitrosyl heme and hexanal concentrations after air exposure for five days at 4°C in cured meat added with plant extracts<sup>1</sup>

Processed meat	Extract concentration % (w/w)	Oxygen radical absorbance capacity (Trolox eq.µmol/100g)	Nitrosylated Heme (mg/kg)	Hexanal (mg/kg)
DCNO	/	10.4 - 11.1 <sup>2</sup>	97 - 100	5-6
DCNO + White grape	0.055	25.8 - 27.3 **	97 - 101	1
DCNO + Carnosic acid	1	30.7 - 31.2 **	95 - 101	<1-1
DCNO + Red wine	2	22.0 - 27.0 *	123 – 138 *	<1-1
DCNO + Pomegranate	0.6	20.1 - 20.5 **	108 - 108	<1
DCNO + α-tocopherol	0.045	12.4 - 12.7 *	91 - 94	<1-1
DCNO without erythorbate	/	9.8 - 11.2	96 - 101	3-8

<sup>1</sup> Plant extracts were added during the curing process to DCNO (dark meat, cured with sodium nitrite, cooked and oxidized).

<sup>2</sup> Data show results of two measures per processed meat batch. Details: see Materials and Methods. \* p=<0.05, p<0.003\*\* compared with DCNO group. Student t test statistics could be done because the within-pair correlation was high, however, P values should be taken cautiously (DeWinter J.C.F. "Using the Student's t-test with extremely small sample sizes" Practical Assessment, Research & Evaluation, 2013, 18, 1-12)